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(57) Abstract

Methods and recombinant cells are provided for increasing the resistance of hematopoietic progenitor cells to the toxic effects of chemotherapeutic and other therapeutic agents used in treating solid tumors and other malignancies. Hematopoietic progenitor cells are obtained from suitable bone marrow donors. The cells are genetically altered by introduction, and stable incorporation into the genome, of at least one DNA segment encoding a protein capable of increasing intracellular glutathione production, thereby imparting to the cells the capability of producing increased amounts of intracellular glutathione. The genetically altered hematopoietic progenitor cells are introduced into a patient for whom chemotherapy has been prescribed. The patient may then be treated with dosages of chemotherapeutic agents that would be toxic to the patient's non-recombinant hematopoietic progenitor cells.

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METHOD AND RECOMBINANT CELLS FOR PROVIDING INCREASED RESISTANCE OF HEMATOPOIETIC PROGENITOR CELLS TO TOXICITY EFFECTS OF CHEMOTHERAPEUTIC AGENTS

FIELD OF THE INVENTION

The present invention relates to cancer chemotherapy. In particular, methods and recombinant cells are provided for increasing cellular resistance of hematopoietic progenitor cells to toxic effects of chemotherapeutic agents used in treating patients having malignant tumors.

BACKGROUND

Several forms of malignant tumors, including ovarian cancer, are effectively treated by anti cancer drugs, including alkylating agents and platinum-containing drugs. Unfortunately, the initial positive response to therapy is often limited by development of resistance to these chemotherapeutic agents. Resistance in ovarian cancer is especially broad, and these tumors often become refractory to other anti-cancer drugs and to radiation. See Ozols et al., Seminars in Oncolog., 18: 222-32 (1991). Chemotherapy at this point is lost as a treatment because the dosages required to affect resistant tumors is pretoxic to normal cell types, particularly hematopoietic stem cells of the bone marrow.

Resistance to radiation, alkylating agents and platinum analogs has been associated with increased cellular glutathione. This is consistent with the known role of glutathione in protecting cells against foreign compounds and the effects of radiation.

The cellular capacity for glutathione synthesis may affect glutathione-mediated resistance to these agents. Glutathione is synthesized intracellularly by the successive actions of γ-glutamylcysteine synthetase and glutathione synthetase. γ-glutamylcysteine synthetase catalyzes the rate-limiting step in glutathione synthesis. Another enzyme, γ-glutamyltranspeptidase, functions in cellular recovery of cysteine moieties, which are required for continued glutathione synthesis. Thus, these two enzymes are essential catalysts in glutathione metabolism. See generally, Meister and Anderson, Ann. Rev. Biochem., 52: 711-60 (1983).

It has been shown that glutathione is associated with cellular resistance to radiation and DNA reactive drugs (platinum analogs and classical alkylating agents). Treatment of resistant tumor cells with D,L-buthionine-(S,R)-sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, J. Biol. Chem., 254: 7558-60 (1979)), partially reverses resistance of cells to such drugs and to radiation. These findings suggest that glutathione plays a significant role in resistance to certain classes of chemotherapeutic agents, as well as radiation therapy, used in treatment of solid malignant tumors.

Glutathione-mediated drug resistance is but one of several types of drug resistance arising from exposure of cells to various agents. For example, resistance to neomycin and analogs thereof is mediated in bacteria by neomycin phosphotransferase. Southern et al., J. Mol. Appl. Gen., 1: 327-41 (1982). Methotrexate resistance appears to be associated with dihydrofolate reductase activity. Classical multiple drug resistance in many cell types has been found to

involve the *mdr*1 gene. These cancerous cells acquire resistance to drugs, such as colchicine, vinblastine and adriamycin, by repeated exposure, which results in enhanced expression of the multiple drug resistance gene. The human MDR1 gene has been found to encode a 4.5 kb mRNA which is overexpressed in resistant cell lines. This mRNA encodes a drug efflux pump glycoprotein (P-glycoprotein), which is believed to function in removal of the cytotoxic drugs from cells. The above examples tend to indicate that resistance to specific drugs is mediated by a variety of different mechanisms.

Although the development of resistance to anti-cancer drugs by tumors is a major problem in cancer therapy, genes encoding drug resistance factors also have potential utility in cancer treatment. For example, it has been reported recently that a cloned mouse MDR gene, when introduced via an expression vector into cultured tumor cells, is sufficient to confer multi-drug resistance to those cells. Gros et al., Nature, 323: 728-31 (1986). Furthermore, a full length cDNA of the human MDR1 gene is capable of conferring multi-drug resistance when integrated into the genome of drug-susceptible recipient cultured tumor cells. NTIS Publication No. PB87-218434, filed June 16, 1987. Moreover, the expression of a human MDR1 cDNA in the bone marrow of transgenic mice conferred resistance to daunomycin, a drug that induces bone marrow toxicity. Galski et al., Molec. and Cellular Biol., 9: 4357-63 (1989). Therefore, it is possible to use genes found to confer drug resistance in tumors to impart similar drug resistance in selected normal cell types, such as hematopoietic progenitor stem cells comprising bone marrow, thus

protecting these cells from unwanted toxic effects of marrow-toxic chemotherapeutic agents.

Currently, bone marrow toxicity is the main dose-limiting factor for many commonly used anti-cancer drugs. The ability to impart drug resistance selectively to bone marrow stem cells would be particularly advantageous in cancer therapy because it would enable dosages of various chemotherapeutic agents to be increased, thereby enhancing the efficacy of chemotherapy without compromising viability of hematopoietic progenitor cells. This may be accomplished by genetically altering the cells to produce more of the protein or other factors responsible for conferring drug resistance. For such a procedure to be useful in clinical applications, however, genes encoding factors involved in drug resistance must be efficiently transferred to isolated human hematopoietic progenitor cells, so that the resultant cells exhibit drug resistance upon infusion into a patient requiring therapy.

Because development of drug resistance is controlled by a variety of mechanisms, success in imparting cellular resistance to one type of drug does not ensure a reasonable expectation of similar success in imparting resistance to another type of drug. For example, the above-described findings relating to classical multi-drug resistance mediated by MDR1 suggest that genetic alteration of bone marrow cells with MDR genes may have clinical applicability for certain classes of drugs, but not for classical alkylating agents or platinum-containing drugs, inasmuch as resistance to platinum-containing and other alkylating agents involves an entirely different mechanism that is independent of MDR1. Thus, methods for conferring glutathione-mediated drug resistance

have heretofore not been available and cannot be inferred from the prior MDR findings.

Given the frequency with which platinum-containing and other alkylating chemotherapeutic agents are used in the treatment of many classes of malignancies, a need exists to develop procedures by which hematopoietic progenitor cells of bone marrow may be made less sensitive to such agents, thereby enabling their use in chemotherapy at higher dosage levels. Such procedures would greatly enhance the effectiveness of solid tumor chemotherapy, including treatment of ovarian cancer by allowing dose escalation.

SUMMARY OF THE INVENTION

In accordance with the present invention, glutathione-mediated resistance to various chemotherapeutic agents may be imparted to drug-sensitive cells by genetically altering the cells to increase their intracellular production of reduced glutathione (GSH). According to one aspect of the present invention, a method is provided for increasing the resistance of hematopoietic progenitor cells to chemotherapeutic agents used in treating a patient having a malignant tumor. According to this method, healthy hematopoietic progenitor cells are obtained from suitable donors. These cells are genetically altered by introducing into the cells, for stable incorporation in the genome, at least one segment of DNA that encodes at least one protein capable of increasing the intracellular production of glutathione in the cells. The genetically altered cells are then infused into a recipient patient for whom chemotherapy has been prescribed. These genetically altered hematopoietic cells and their progeny possess enhanced

resistance to chemotherapeutic agents that would be toxic to hematopoietic progenitor cells not genetically altered in this manner. For this reason, a patient requiring anti-cancer chemotherapy may be treated with larger doses of chemotherapeutic agents than would normally be tolerable due to bone marrow toxicity.

According to another aspect of the present invention, the above-described method specifically comprises providing healthy hematopoietic progenitor cells, preferably from a cancer patient prescribed to receive chemotherapy with drugs whose dosage is limited by bone marrow toxocity. The cells are then genetically altered by infection with a replication-incompetent retroviral shuttle vector which comprises at least one cDNA encoding a biologically active protein capable of increasing intracellular glutathione production. In a preferred embodiment, the cDNA encodes an enzymatically active, preferably human, γ-glutamylcysteine synthetase or a cDNA encoding an enzymatically active glutamyltranspeptidase. The cDNA becomes stably incorporated into the genome of the hematopoietic progenitor cells, thereby imparting to the cells the capability of producing increased amounts of intracellular glutathione. These cells are infused into a patient requiring chemotherapy, such that increased dosages of chemotherapeutic agents may be administered without the bone marrow toxicity that would result without genetically altering the hematopoietic progenitor cells.

According to another aspect of the present invention, the types of chemotherapeutic agents that may be administered in increased dosages in the practice of the present invention include platinum-

containing agents such as cisplatin and carboplatin, classical bifunctional alkylating agents such as melphalan, subclasses of natural products such as anthracyclines and epipodophilotoxins and compounds such as vinblastine. In addition, radiation therapy may be administered in increased dosages, either alone or in combination with the chemotherapeutic agents such as those mentioned above, in accordance with the practice of the invention.

According to a further aspect of the invention, recombinant hematopoietic progenitor cells are provided for use in conjunction with chemotherapeutic agents and/or radiation therapy in treatment of patients having a malignant tumor. These cells comprise a genome having at least one introduced segment of DNA that encodes at least one protein capable of increasing intracellular production of glutathione. Thus, these cells have enhanced resistance to concentrations of chemotherapeutic agents that would be toxic to non-recombinant hematopoietic progenitor cells.

According to yet another aspect of the invention, recombinant hematopoietic progenitor cells are provided which comprise a genome containing at least one introduced segment of DNA that encodes an expressible, enzymatically active form of γ-glutamylcysteine synthetase capable of increasing intracellular production of glutathione. These cells express γ-glutamylcysteine synthetase in an amount effective to substantially negate the inhibitory effect of a standard inhibitory dosage of BSO, which as noted above, is a specific inhibitor of γ-glutamylcysteine synthetase. These cells have enhanced resistance to normally toxic concentrations

of chemotherapeutic agents used for treatment of malignant tumors.

According to yet another aspect of the present invention, a method is provided for treating a patient having a malignant tumor by administration of chemotherapeutic agents used in treatment of such tumors. This method comprises infusing into the patient recombinant hematopoietic progenitor cells comprising a genome containing a DNA segment that encodes an expressible, enzymatically form of γ-glutamylcysteine synthetase capable of increasing intracellular production of glutathione. The γ-glutamylcysteine synthetase is expressed in an amount sufficient to substantially negate the inhibitory effect of a standard inhibitory dosage of BSO and the cells have enhanced resistance to normally toxic concentrations of the above-mentioned chemotherapeutic agents. Following infusion, the method further comprises administering to the patient at least one of the above-mentioned chemotherapeutic agents at a dosage that would be toxic to the patient's non-recombinant hematopoietic progenitor cells.

Practice of the present invention provides several notable advantages, which, insofar as is known, were previously unavailable in cancer chemotherapy. The failure to diagnose cancer early in its clinical course at a time when it is amenable to complete surgical resection leaves a patient with but one hope for cure. The tumor must be completely irradicated by the use of anti-cancer drugs, irradiation and/or biological therapy. Some types of tumors show excellent responses when subjected to aggressive chemotherapy (e.g., ovarian cancer). However, tumors frequently recur and are refractory to further chemotherapy at the maximum dosage levels

which currently may be used. Increased dosages, which could provide effective irradiation of these refractory solid tumors or the ability to treat initially with higher dosages, could not be administered heretofore because of the resultant toxicity to normal tissues, including hematopoietic stem cells present in bone marrow. The present invention provides a way to increase the resistance of bone marrow stem cells to these chemotherapeutic agents, thereby permitting administration of increased dosages which could effectuate a cure. This strategy has particularly significant implications in the treatment of advanced solid tumor cancers, where high-dosage chemotherapy may be the only treatment available. The methods set forth in the present invention provide a potentially life-saving therapeutic treatment for patients suffering from cancer.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has now been discovered that ovarian tumor cell lines demonstrating high resistance to the platinum-containing chemotherapeutic agent, cisplatin, as well as cross resistance to carboplatin and melphalan, possess markedly elevated levels of reduced glutathione (GSH), and a close correlation exists between drug resistance and the level of GSH present in resistant cell lines. GSH elevation in the cell lines corresponds to a steady state increase in the amount of messenger RNA encoding two enzymes involved in the glutathione biosynthetic/metabolic pathway: γ-glutamylcysteine synthetase (γ-GCS), which catalyzes the controlling (and feedback-inhibited) steps of GSH synthesis, and γ-glutamyltranspeptidase (γ-GT), which

functions in cellular recovery of cysteine moieties (salvage pathways). Resistance of the above-described ovarian cancer cell lines is reduced when the cells are treated with BSO, a specific inhibitor of γ-GCS. Griffin and Meister, J. Biol. Chem., 254: 7558-60 (1979). Moreover, clinical trials indicate that ovarian cancer patients receiving inhibitory doses of BSO respond with decreased amounts of GSH in tumor cells.

A hematopoietic progenitor cell (i.e., stem cell) may be defined as a pleuripotent cell that gives rise to progeny in all defined hematolymphoid lineages. Stem cells have the ability to restore, when transplanted, the production of hematopoietic and lymphoid cells to an individual who has lost such production due, e.g., to immunocompromising disease, radiation or chemotherapy. It is believed that if glutathione-mediated resistance to alkylating chemotherapeutic agents is imparted to hematopoietic progenitor cells, larger amounts of such agents could be used in chemotherapy of tumors without resulting in dose-limiting bone marrow cytotoxicity. In accordance with the present invention, a cDNA encoding human γ-GCS is isolated and cloned into a mammalian expression vector, pLTR-2. As will be described more fully below, transfection of drug-sensitive cell lines with the γ-GCS cDNA-containing vector may impart to these cell lines resistance to various alkylating chemotherapeutic agents and irradiation. Resistance is correlated with expression of the recombinant γ-GCS protein and with intracellular production of GSH. Thus, genetic alteration of cell lines with a key regulatory enzyme involved in glutathione biosynthesis can confer resistance to previously drug-sensitive cells. In accordance with the present invention,

other enzymes involved in the biosynthesis and metabolism of glutathione, either alone or in combination are contemplated in conferring or enhancing bone marrow resistance to several classes of anti-cancer drugs.

The description which follows sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes for illustration and is not intended to limit the invention. Unless otherwise specified, standard cloning and recombinant DNA methods, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") are used.

I. Sources of, and Methods for Obtaining and Replacing Hematopoietic Progenitor Cells

A. Preparation of Recipient Cells

Bone marrow, comprising hematopoietic progenitor cells (sometimes referred to herein as stem cells), may be obtained from autologous, allogeneic or syngeneic donors by methods well known to physicians and others skilled in the art. See, e.g., E. Beutler, Clinical Bone Marrow Transplantation, K. G. Blume and L. D. Petz, eds., Churchill Livingstone, pp. 1-13 (1983). Briefly, bone marrow for transplantation is obtained by multiple aspirations from the iliac crest of a donor. The aspirate is mixed with an anticoagulant (e.g., heparin) and a tissue culture medium. Following coarse filtration, bone marrow cells are subjected to any desired manipulations, such as those described in further detail below. Hematopoietic progenitor cells may be further purified from the bone marrow aspirate, if desired, as

described herein. The bone marrow cells are then infused intraveneously into the recipient (i.e., a patient requiring bone marrow transplantation). The cells migrate through the bloodstream to the recipient's bone marrow, where they establish colonies and continue to divide.

II. Sources and Preparation of DNA Encoding Proteins Involved in Glutathione Biosynthesis/Metabolism

A. DNA Segments Encoding Glutathione-Affecting Enzymes

Any DNA segment encoding a biologically active protein involved in the synthesis and/or metabolism of glutathione has potential utility in the practice of the present invention. Human cDNAs are preferred, though genes may also be useful in some cases. In a preferred embodiment, a cDNA encoding an enzymatically active γ-glutamylcysteine synthetase (γ-GCS) is employed. In an alternative preferred embodiment, a cDNA encoding enzymatically active γ-glutamyltranspeptidase is utilized, either alone or in combination with γ-GCS. DNA segments encoding other glutathione-regulating enzymes may also be used, either alone or in combination. These include DNA segments encoding: glutathione synthetase, glutamine synthetase, γ-glutamylcyclotransferase, 5-oxoprolinase, glutathione peroxidase, glutathione reductase and glutathione transhydrogenase. cDNAs encoding the above-mentioned enzymes are obtainable by a variety of methods known in the art. For example, an appropriate cDNA library (e.g., human, in a preferred embodiment) may be screened with heterologous cDNAs or homologous cDNA fragments, if available, or oligonucleotide probes, if suitable

sequence information is available. Alternatively, cDNA expression libraries may be screened with antibodies raised against the glutathione-affecting enzyme of interest. See, e.g., Huynh et al., DNA Cloning: A Practical Approach, Vol. 1, D. M. Glover, ed., pp. 49-78 (1985) for general methods of screening a lambda gt 11 expression library. Similarly, genomic libraries may be screened with DNA probes or antibodies; however, cDNAs, because of their size and lack of introne containing repetitive sequences, are more likely to be useful in the practice of the present invention.

B. Vectors and Producer Cell Lines

DNA segments encoding the above-mentioned glutathione-affecting proteins are utilized for genetically altering hematopoietic progenitor cells by cloning into appropriate vectors. Retrovirus-mediated DNA transfer is a preferred method in the practice of the present invention. Retrovirus-mediated DNA transfer is defined as a method by which replication-defective retroviruses and retroviral vectors are used to transfer segments of foreign DNA into host cells infectable by such viruses, and to effect stable integration of those DNA segments into the genomes of such host cells. The use of retroviral vectors is the method of choice because of the high efficiency of gene transfer that may achieved. Exogenous genes carried within the retrovirus genome are brought into target cells by the virus. Once in the cell, these genes integrate into the host cell chromosome along with the viral DNA and are expressed. retroviral vectors have been constructed so they do not contain any viral protein-encoding genes but only the desired exogenous genes. The missing viral functions (i.e., the synthesized *gag*, *pol*, and *env* proteins) are

provided in *trans* by packaging cell lines that act as helper viruses, but cannot themselves propagate. Thus, only the desired genes enter the host cell and further virus spread is not possible. Retroviral mediated gene transfer has been applied successfully for the insertion and expression of several different genes in hematopoietic stem cells of several mammalian species; thus, methods for accomplishing such gene transfer are well known in the art.

Examples of suitable retroviral shuttle vectors are the SVX-neo (Cepko et al., Cell, 37: 1053-62 (1984)) and the N2 (Keller et al., Nature, 318: 275-77 (1985)) vectors and derivatives thereof. SVX-neo is a highly transmissible replication-defective retroviral shuttle vector. This vector consists of the Moloney murine leukemia virus (MoMuLV) from which the viral structural genes have been removed, leaving both long terminal repeat sequences (LTR), the retroviral encapsidation signal sequence (Psi), a portion of the *gag* coding sequence, including the splice donor and the splice acceptor sites, and the SV40 and pBR322 ori sequences. The bacterial gene Neo^R (derived from the TN5 transposon) conferring resistance to the antibiotic neomycin (or its analogue G418 that is toxic to mammalian cells) has been inserted as a dominant selectable marker. In accordance with the present invention, this vector (referred to as SVX/SV40) has been modified so that it contains two promoters, the viral LTR and the SV40 early promoter, in opposing transcriptional orientations. The Neo^R gene is driven by the retroviral 5' LTR, and the cDNA encoding the enzymatically active subunit of γ-GCS is driven by the SV40 early promoter.

Additional DNA segments described above may be substituted for γ-GCS into the SVX/SV40 vector for transfer into bone marrow.

Safe DNA transfer into mammalian cells using retroviral vectors requires the availability of safe amphotrophic retrovirus packaging cell lines, which are incapable of producing wild type virus. Examples of appropriate producer cell lines, capable of safely and efficiently mediating retroviral gene transfer are well known in the art. For example, an amphotropic packaging cell line, "GP+ *env* Am-12", in which the viral *gag* and *pol* functions are present in one plasmid, and the *env* function on another plasmid, is disclosed by Markowicz et al., Virology, 167: 400-06 (1988). Additionally, a high-titer retrovirus producer cell line, capable of mediating gene transfer into primate hematopoietic stem cells, has been disclosed, and may be used advantageously in the practice of the present invention. Bodine et al., Proc. Natl. Acad. Sci. (USA), 87: 3738-42 (1990). This producer cell line, which secretes gibbon IL-3 and human IL-6 and produces greater than 10^{10} functional viral particles per ml of culture medium, has been used to reproducibly transfer genes into bone marrow stem cells of rhesus monkeys.

Though high-titer replication-incompetent retrovirus-mediated DNA transfer is the preferred means of genetically altering hemotopoietic progenitor cells, other vectors may also be useful. For example, as described more fully in the following examples, cDNAs or genes encoding the above-mentioned glutathione-affecting proteins may be cloned into mammalian expression vectors, then utilized to transfect cultured mammalian cells. For example, to derive cell lines overexpressing γ-GCS, a retroviral

long terminal repeat (LTR) is placed 5' to a full length γ-GCS cDNA. This construct can be used with pKOneo, a plasmid encoding resistance to the neomycin analog, G418, to co-transfect cultured mammalian cells, e.g., ovarian tumor cells. Transformed cells are selected by resistance to G418 and verified for genomic integration and expression through DNA, RNA and protein blotting. Thus, depending on the type of vector used, genetic alteration of hematopoietic progenitor cells may be accomplished by means of transfection (via standard mammalian expression vectors) or high-efficiency infection (via replication-incompetent retroviral vectors).

C. **Promoters**

Recombinant vectors should be constructed so that DNA segments encoding the above-described glutathione-affecting proteins are positioned downstream from a viral or cellular promoter, thereby enabling high expression of the DNA segment. Many constitutive and inducible promoters are known in the art, and may be suitable for expression of recombinant proteins in genetically altered hematopoietic stem cells.

In a preferred embodiment, the expression vector features a hormone inducible promoter, the mouse mammary tumor virus long terminal repeat, which controls the expression of a cDNA encoding γ-GCS. This vector also features the neo*R* (neomycin phosphotransferase) gene under the control of the MoMuLV LTR. Other promoters contemplated for use in the present invention include a human IL3 inducible promoter. Constitutive promoters which may be used to advantage include a human γ-globin promoter, a β-actin promoter, as well as others.

III. Methods for Genetically Altering Hematopoietic Progenitor Cells

A. *In Vitro* Determination of Effectiveness of Selected Genetic Alterations

High-efficiency retrovirus-mediated DNA transfer, for the purpose of genetically altering hematopoietic stem cells, may be accomplished by infection protocols known in the art. See, e.g., Bodine et al., Proc. Natl. Sci. (USA), 87: 3638-42 (1990). However, before a selected DNA segment can be used in clinical application, its effectiveness in increasing intracellular glutathione should be determined. This may be accomplished by introducing, e.g., by transfection, mammalian expression vectors containing selected DNA segments into cell lines with low levels of GSH. Genetic alteration may also be accomplished by other methods known in the art, such as calcium phosphate precipitation, liposome-mediated transfer or electroporation. Genetically altered cultured cell lines may then be tested for the presence of the selected DNA segment, as well as its expression, by standard methods including DNA blotting, RNA blotting and immunoblotting. Suitably altered cell lines should be analyzed for overproduction of intracellular GSH. In a preferred embodiment, a cDNA encoding γ-GCS is used to genetically alter cisplatin-sensitive cell lines. Overexpression of γ-GCS in these cell lines may be tested by treatment with the specific γ-GCS inhibitor, BSO. If overexpression of γ-GCS has been achieved in these cell lines, they should be able to produce elevated levels of GSH, even when treated with standard inhibitory doses of BSO (e.g., 25-100 μM). This effect (i.e., production of elevated levels of

GSH in the presence of 25-100 μM BSO) is sometimes referred to herein as substantial negation of the inhibitory effect of a standard inhibitory dosage of BSO.

For transfection of cultured cells with expression vectors containing selected DNA segments, the following procedure may be followed. Exponentially growing cells are trypsinized and seeded at a density of 5 x 10^5 cells/10 ml dish and grown overnight at 37°C. Cells are washed twice with serum free RPMI medium and re-fed with 7 ml of the medium prior to transfection. 10 micrograms of plasmid DNA (pLTR-2/γ-GCS, as described above) and 1 microgram of pKO neo (as described above) in a total volume of 50 microliters of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) are mixed with 50 microliters of Lipofectin Reagent (Gibco BRL) and incubated at room temperature for 15 minutes. The mixture is then added dropwise to the cells, and after an overnight incubation at 37°C, the cells are fed with an equal volume of RPMI medium supplemented with 20% fetal bovine serum, then incubated for another 48 hours. The cells are re-fed with RPMI medium containing the neomycin analog, G418, at a concentration of 500 μg/ml. Individual G418 resistant clones are picked 2-3 weeks later.

After overexpression of the selected glutathione-affecting enzyme (e.g., γ-GCS) has been confirmed by RNA and protein blotting, cellular glutathione levels and resistance to cytotoxicity of selected alkylating chemotherapeutic agents is determined. Methods for making these determinations are described in the examples below.

Cell lines overexpressing γ-GCS are further tested with the γ-GCS inhibitor, BSO, according to the following general procedure. Potentially cytotoxic

agents, such as melphalan, cisplatin or radiation, are administered to the transfected cell line in the presence or absence of γ-GCS-inhibitory concentrations of BSO (e.g., 25-100 μM). Procedures for testing transfected cell lines using BSO are more fully set forth in the examples below. Cell lines that overexpress γ-GCS will show higher resistance to the cytotoxic agents than cell lines which were not genetically altered to overproduce γ-GCS. Hematopoietic progenitor cells that have not been genetically altered in accordance with the present invention are sometimes referred to herein as non-recombinant hematopoietic progenitor cells.

B. High-Efficiency Retrovirus-Mediated DNA Transfer

Once it has been determined that a particular DNA segment encoding a glutathione-affecting protein is effective in increasing intracellular glutathione production in cultured cells, it may be developed for high-efficiency infection of hematopoietic progenitor cells. The DNA segment of interest may be cloned into replication-defective retroviral shuttle vectors by the methods mentioned above. Healthy, hematopoietic progenitor cells are obtained from suitable donors, including autologous donation from a patient for whom chemotherapy has been prescribed, also as described above.

According to Bodine et al., *supra*, the following steps are performed. Producer cells, as described above in Section II, are grown to confluence. On the first day of the infection protocol (Day 1), confluent plates of producer cells are split 1:10 in DMEM/10% fetal calf serum (FCS) containing 6 micrograms/ml Polybrene (Sigma Chemical

Co.). On the same day, bone marrow is aspirated from donor individuals as described in the preceding section, into DMEM/2% FCS containing 10 units/ml heparin. Mononuclear cells are isolated from the bone marrow by centrifugation through a lymphocyte separation medium, e.g., Organon Teknika-Cappel, according to the supplier's instructions. Approximately 1-2 x 10^7 mononuclear cells are added to each plate of producer cells, and co-cultivation is allowed to proceed for three days. During this time, the recombinant retrovirus vectors, containing the DNA segments of interest, are transferred from the producer cells to the mononuclear stem cells by infection. The replication incompetent vector then becomes stably integrated with the genome of the recipient cells. On Day 3, an additional quantity of confluent producer cell plates are split 1:10 in DMEM/10% FCS. The non-adherent cells are collected from the original plates and reseeded on the fresh plates for an additional three days. On Day 6, the non-adherent co-cultivated mononuclear cells are collected, washed in Hanks' balanced salt solution and infused into the recipient in 50 ml of, e.g., phosphate buffered saline containing heparin at 10 units/ml. Human IL-3 (approximately 200 units/ml) may be added to the cultures to facilitate colony development after reinfusion.

IV. Administration of Chemotherapy

Patients who have been infused with recombinant hematopoietic progenitor cells, according to the methods described above, are capable of withstanding higher dosages of several chemotherapeutic agents effective in the treatment of solid tumors. Such chemotherapeutic agents include,

but are not limited to, platinum-containing agents such as cisplatin and carboplatin, classical bifunctional alkylating agents such as melphalan and cyclophosphamide, natural products subclasses including anthracyclines and epipodophilotoxins, as well as vinca alkaloids such as vinblastine. Increased dosages of radiation therapy may also be administered to patients treated in accordance with the present invention. The amount to which these dosages may be increased for an individual chemotherapeutic agent may be determined by *in vitro* testing of cultured cells as described in Part III-A above. These elevated dosages may be tested *in vivo* in animal model systems, according to common methods known in the art. Such a method is described in detail in the examples below. Once it has been determined that a selected genetic alteration, such as insertion of a γ-GCS-encoding cDNA in the bone marrow cell genome, allows a particular chemotherapeutic agent to be administered at a known increased rate, the method may be applied clinically to human patients. Dosages may be adjusted to individual patients, using methods routine to pharmacologists and physicians.

The following examples are provided to describe the invention in further detail. These examples are intended merely to illustrate and not to limit the invention.

Example 1

Transfection and Expression of a cDNA Encoding γ-GCS in Cultured Ovarian Cancer Cells

A. Transfection

Two ovarian cancer cell lines, A2780 and 1A9, have been determined suitable for transfection/

expression of γ-GCS, as it has been established, by methods described herein, and routine methods known in the art, that both of these cell lines possess very low levels of endogenous glutathione (relative to a number of cell lines examined), express limited amounts of γ-GCS, GSH synthetase, and γ-GT, and are highly sensitive to cisplatin and its related analogs. It has also been determined that A2780 and 1A9 cells are readily "transfectable", which will allow a number of clonal cell lines carrying the desired constructs to be obtained.

To derive cell lines overexpressing γ-GCS, a retroviral LTR is placed 5' to the γ-GCS cDNA. This construct is used with pKOneo, a plasmid encoding resistance to the neomycin analogue, G418, in co-transfection with A2780 cells. Clonal populations are selected and verified for genomic integration and expression through combination of DNA (Southern), RNA (Northern) and protein (Western) blotting. Each clone is expanded, the amount of γ-GCS quantitated and the cells examined for increased intracellular levels of GSH and for altered sensitivity to plantinum drugs, alkylating agents and irradiation. As a control, these cell lines are compared to the parental A2780 cell line and a vector (pLTR-2 and pKOneo)-only G418 resistance transfectant.

Expression Vector: A full length cDNA clone for γ-GCS was obtained from a library prepared from highly cisplatin resistant human ovarian tumor cells. A NotI fragment of the γ-GCS cDNA, corresponding to the 5' untranslated region, the coding region, and 251 nucleotides of the 3' untranslated region, may be subcloned (by blunt-end ligation) into the Bam HI site of the expression vector pLTR-2. This expression vector utilizes the long terminal repeat (LTR) of the

Moloney murine leukemia virus to drive transcription and has successfully been used to express a number of other cDNAs in the ovarian tumor cell line, A2780. There has also been constructed an expression vector which features an inducible promoter (the heavy metal-inducible mouse metallothionein I promoter), the SV40 polyadenylation signal, and the bacterial neomycin phosphotransferase gene under the control of the SV40 late promoter.

Transfection: Exponentially growing cells are trypsinized and seeded at a density of $5x10^5$ cells/10mm dish and grown overnight at 37°C. The cells are washed twice with serum free RPMI medium and refed with 7 mls of RPMI medium prior to transfection. 10 μg of plasmid DNA (pLTR-2/γ-GCS) and 1 μg of pKOneo (a plasmid containing the gene for geneticin (G418, Gibco BRL) resistance) in a total volume of 50μl of TE (10 mM Tris-HCl, 0.1 mM EDTA) are mixed with 50μl of Lipofectin Reagent (Gibco BRL) and incubated at room temperature for 15 minutes. The mixture is then added dropwise to the cells and after an overnight incubation at 37°C, the cells are fed with an equal volume of RPMI medium supplemented with 20% fetal bovine serum, and incubated for another 48 hours. The cells are refed with RPMI medium containing G418 at a concentration of 500μg/ml. Individual G418 resistant clones are picked 2 to 3 weeks later using sterile cotton tipped applicators and expanded. Lipofectin Reagent mediated transfection of A2780 cells has provided more efficient transfection than the standard method of Graham and van der Eb employing calcium phosphate. Transfected cell lines are checked for integration by standard Southern blotting techniques.

B. Analysis of Expression

Extraction of Cellular RNA and Northern Blotting: Expression of the transfected γ-glutamylcysteine synthetase cDNA may be evaluated by Northern blot analysis. Total cellular RNA is extracted by a modified one step guanidinium isothiocyanate-phenol-chloroform extraction procedure. Total RNA (16 μg per lane) is denatured in 50% formamide containing 7.4% formaldehyde and separated by electrophoresis on an agarose (1% agarose/2.2M formaldehyde) gel. The RNA is blotted by capillary action on to Magna NT membrane filters (Micron Separations, Inc., Westboro, MA) in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and hybridized (>12h) with ^{32}P-labeled probes. The γ-GCS cDNA (~3.7 kb) probe is ^{32}P labeled using the Prime-It random primer kit (Stratagene) to a specific activity of >5 x 10^8 dpm/μg. RNA gels are stained with ethidium bromide and examined to ascertain that equivalent amounts of RNA are analyzed. Filters are washed twice for 30 min. each in 2X SSC containing 0.5% SDS and sodium pyrophosphate at 65°C and twice for 30 min. each in 0.2X SSC containing 0.5% SDS and sodium pyrophosphate at 55°C. Autoradiography is performed at -70°C for 1 to 4 days. The expression vector directs the transcription of a mRNA that is ~1.8 kb longer than the cDNA and so the size difference may be used to distinguish it from endogenous mRNA.

Western blot analysis: Total cellular protein is prepared (Furth et al., J. Virol., 43: 294-304 (1982)) and relative protein concentration is estimated fluorometrically (Bohlen et al., Arch. Biochem. Biophys., 155: 213-20 (1973)) using bovine serum albumin (BSA) as a standard. Lysate (100 μg) from cell extracts is mixed with an equal volume of 2X

SDS buffer (40% glycerol, 6% SDS, 0.25M Tris-HCl, 0.1% bromophenol blue, and 12% urea, pH 6.8) containing 2-mercaptoethanol (0.7M), and placed at 100°C for 5 min. The samples are separated by electrophoresis on a 10% acrylamide gel using pre-stained protein molecular weight standards. The separated proteins are transferred electrophoretically to Immobilon-P membrane filters (Millipore Corporation) and washed twice (30 min.) in 50 mM Tris-HCl (pH 7.5), 400 mM NaCl (TBS) containing 0.05% Tween 20 (TBS-T) at 20°C. The filters are preincubated for 90 min. (20°C, with agitation) with blocking solution, TBS-A, (TBS containing 5% nonfat dry milk). The filters are incubated with a polyclonal antibody to γ-glutamylcysteine synthetase (rabbit antirat) diluted 1:250 in TBS-A for at least 12 h. The filters are then washed three times with TBS-T (10 min.) and treated with a secondard antibody (^{125}I-donkey anti-rabbit IgG; diluted 1:300 in TBS-A) for 1 h. After thorough washing with TBS-T, the filters are air dried. Autoradiography is performed at -70°C for 1 week.

Cytotoxicity Assays: Cisplatin, carboplatin, melphalan and BSO sensitivity is determined by the tetrazolium salt assay. Cells (1,000 to 8,000) are plated in 150 μl of RPMI medium per well in a 96-well plate. After incubation overnight, drug is added in varying concentrations (0-300 μM for cisplatin, 0-10 mM for carboplatin, 0-1 mM for melphalan and 0-30 mM for BSO). After 3 days, 40μl of 5 mg/ml 3,(4,5,-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) is added per well. After 2h incubation, the cells are lysed with 100 μl per well of extraction buffer (20% (w/v) sodium dodecyl sulfate, 50% N,N-dimethyl formamide, pH 4.7).

After incubation overnight, the absorbance at 570 nm is measured (Bio Rad Microplate multiscanner) employing the wells without cells as blanks. When cultures are pretreated with BSO, the γ-GCS inhibitor is added as a concentrated solution directly to the culture medium, and the cells are treated 24h later by the addition of cisplatin, carboplatin, or melphalan.

Glutathione Determinations: Cells (2 to 5 x 10^6; about 60% confluent) are lysed by sonication in 1 ml of phosphate buffered saline (PBS) (4°C). The supernatant is obtained for assay after centrifugation (10,000xg, 10 min, 4°C). The protein is precipitated by adding 12% 5-sulfosalicylic acid (SSA) (1 vol SSA to 3 vols of sample). After standing on ice for 1 to 4h, the samples are centrifuged (10,000 g, 10 min). The SSA extract is assayed as described by Griffith, Anal. Biochem., 106: 207-12 (1980) using 100 μl of sample and 0.5 u of glutathione reductase per assay. Protein in the PBS lysate is determined by the Bradford assay (BioRad, Richmond, VA) using BSA as a standard.

γ-Glutamylcysteine synthetase assay: The spectrophotometric assay of Seelig et al., J. Biol. Chem., 259: 9345-47 (1984) is used. This method measures the rate of formation of ADP, which is derived from the decrease in absorbance of NADH (monitored at 340 nm) in a coupled enzymatic reaction. Activity is determined at 37°C in reaction mixtures (final volume, 1.0 ml) containing 0.1 M Tris-HCl buffer (pH 8.0), 150 mM KCl, 5 mM Na_2 ATP, 2mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM Na_2 EDTA, 0.2 mM NADH, 17 μg pyruvate kinase, and 17 μg lactate dehydrogenase. The reaction is initiated by the addition of sample containing γ-glutamylcysteine

synthetase. One unit of enzyme activity is defined as the amount that results in the formation of 1 μmol of product per hour at 37°C, and expressed as nmol/min. Specific activity is expressed on the basis of protein determined by the Bradford assay (Bio-Rad).

Metabolic Labeling and Immunoprecipitation of γ-GCS Protein: Cells are labeled in 60-mm Nunc tissue culture dishes by refeeding logarithmically growing cultures with 2 mls of methionine-free RPMI 1640 (Sigma) containing 10% dialyzed fetal calf serum (Gibco) and 100μCi/ml [^{35}S]-methionine (~1000Ci/mmol, NEN, DuPont). Labeling is carried out at 37°C for between 3 and 24 hours and the cell lysates are prepared as follows. The radioactive medium is removed, and the cell monolayer is washed twice in ice cold 1xPBS. The cells are scraped from the plate into 2 mls of PBSTDS buffer (10 mM dibasic sodium phosphate, 7.2, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.2% sodium azide, 0.004% sodium fluoride (pH 7.25)) at 4°C. Tissue culture dishes are rinsed with an additional 1 ml of ice cold PBSTDS and combined with original extract. The cells are incubated at 4°C for 10 minutes followed by vigorous vortexing to facilitate lysis, and the extracts are centrifuged for 1 hour at 35,000 rpm (100,000xg) in Beckman 75Ti at 4°C. The supernatant (S100) is recovered and either used immediately or stored at -70°C.

Each immunoprecipitation reaction contains approximately 1x10^7 trichloroacetic acid-precipitable cpm of [^{35}S]-labelled cell extract. Immuno-precipitation is carried out in PBSTDS. Each 300 μl reaction mixture contains labeled extract and 10 μl of polyclonal rabbit anti-rat γ-glutamylcysteine synthetase antibody. The reaction is incubated at 4°C

for 4 hours, samples are constantly rotated during the incubation time. To render insoluble the antigen-antibody complexes, 100 μl of 10% (vol/vol) protein-A agarose is added, and the tube is rotated overnight at 4°C. The immunoprecipitates are collected by centrifugation and washed extensively, 5 to 6 times in PBSTDS. Immunoprecipitates are dried, dissolved in 1X SDS sample buffer (see Western Blot Analysis) and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Protein bands are identified by fluorography utilizing EN^{3}HANCE (NEN, DuPont).

Example II

Animal Model Systems to Determine Dosage of Chemotherapeutic Agents

Once a DNA segment encoding a glutathione-affecting protein has been determined to be effective *in vitro* in increasing production of intracellular glutathione in cultured, transfected cell lines, the glutathione-mediating effect of genetically altering bone marrow stem cells with that DNA segment may be tested *in vivo*. Various animal model systems for such *in vivo* testing are known in the art. Examples are set forth hereinbelow.

A. Mice

Mice may be used to determine *in vivo* dosages of chemotherapeutic agents, in the practice of the present invention. See, e.g., Dunbar et al., Oncogene Research, 6: 39-51 (1991); Bodine et al., Proc. Nat'l. Acad. Sci. (USA), 86: 8897-8901 (1989). Mice may be obtained from any commercial source. 6- to 10-week-old mice are suitable for *in vivo* testing. Mice may be divided into four suitable test populations: (i) untreated (control); (ii) bone marrow removed and replaced without further

manipulation; (iii) bone marrow removed and cells infected with retroviral vectors containing no DNA segments encoding glutathione-affecting proteins; and (iv) bone marrow removed and infected with retroviral vectors containing DNA segments encoding glutathione-affecting proteins.

Bone marrow cells may be harvested from the hind limbs of donor mice 48 hours after they have received 150 mg/kg 5-fluorouracil (5-FU) (Fluka) intraveneously. Preconditioning of the donor marrow with 5-FU, which increases both stem cell cycling and the relative number of stem cells, has been shown to facilitate retroviral gene transfer. Alternatively, hematopoietic cells from mouse fetal liver may be used in these and subsequent protocols. The isolated stem cells are washed and resuspended at a concentration of 5×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM, Biofluids, Rockville, MD) containing 15% (vol/vol) fetal calf serum (FCS, HyClone), 0.3 mg/ml L-glutamine and appropriate growth factors, such as IL-3 (200 units/ml) and IL-6 (20-200 units/ml). 10 ml of cell suspension may be plated on Sarstedt plates and incubated at 37°C (95% air/5% CO_2) for 2 days. Cells may be recovered by centrifugation, quantitated and resuspended in DMEM containing the same appropriate growth factors and 6 micrograms/ml of Polybrene (Sigma). Approximately 1-5 $\times 10^6$ cells may be added to plates containing appropriate producer cells split 1:5-1:10 24 hours previously. Marrow cells and producer cells are co-cultivated 48 hours. Following co-cultivation, the cells are recovered by aspirating the non-adherent cells from the plates, washing, and resuspending in phosphate-buffered saline (PBS). Approximately 1-3 $\times 10^6$ cells are introduced to recipient mice (in this case, an autologous transplant

wherein the donor mouse receives its own marrow cells) by injection through the tail vein. Alternatively, genetically anemic W/W^v mice can be used for these procedures eliminating the need for lethal irradiation; transplantable bone marrow cells competitively repopulate W/W^v mice due to their functional stem cell deficiency.

After successful recolonization of recipient bone marrow (as determined when acceptable neutrophil levels are achieved) with genetically manipulated and unmanipulated bone marrow cells, mice may be subjected to treatment with varying doses of selected chemotherapeutic agents. For example, sample groups of the four above-mentioned test populations may be treated with dosages of carboplatin ranging from 0 to 160 mg/kg body weight. Animals in test populations 1-3 should exhibit significantly greater bone marrow sensitivity to higher doses of carboplatin, while animals from test population 4 should exhibit higher bone marrow resistance. The maximum dose of carboplatin (160 mg/kg), approximately twice the 50% lethal dose of normal mice, is highly toxic to bone marrow of mice and humans resulting in leukopenia. Recipient mice may be observed daily and samples of blood withdrawn for WBC counts, including platelets, and Wright's stained smears before and 1, 3, 8, and 14 days after injection of the various dosages of carboplatin.

In a preferred embodiment, a cDNA encoding γ-GCS is utilized in the above-described *in vivo* test. Elevated resistance of recipients of such genetically altered hematopoietic cells may be further examined through the use of BSO. Animals from test population 4, which will exhibit higher bone marrow resistance to carboplatin because of overexpression of γ-GCS, may be

subjected to treatment with BSO (1500-4500 mg/m^2/q 12h x 6). This should result in transient inhibition in γ-GCS overexpression and a temporary concomitant return to sensitivity of bone marrow to carboplatin. These results will indicate that the recombinant γ-GCS is indeed being expressed *in vivo* in the test animals, thereby conferring a higher level of drug resistance due to elevated production of GSH.

B. Primates

Methods for retrovirus-mediated genetic alteration of primate bone marrow hematopoietic cells is also known in the art. See, e.g., Bodine, Proc. Nat'l. Acad. Sci. (USA), 87: 3738-42 (1990); Kohn et al., Blood Cells, 13: 285-98 (1987). A suitable primate for *in vivo* tests is the rhesus monkey, preferably adolescent, weighing approximately 4 kg. A suitable infection protocol is described supra in the Detailed Description of the Invention. Animals may be divided to 4 test populations, as described for mice above, and subjected to similar manipulations of the bone marrow. After replacement of the appropriately-treated bone marrow cells into recipient monkeys, the animals may be subjected to treatment with varying concentrations of chemotherapeutic agents, e.g., 0-200 mgs/m^2 carboplatin, as described for mice above. Testing of primates in this manner should yield an accurate representation of the dosage range suitable for clinical use in humans.

While certain preferred embodiments of the present invention have been described above, it is not intended to limit the invention to such embodiments, but various modifications may be made thereto without departing from the spirit of the present invention, the full scope of which is delineated by the following claims.

What is claimed is:

1. A method for increasing the resistance of hematopoietic progenitor cells to therapeutic agents used in treating a patient having a malignant tumor, said method comprising:

a) providing healthy hematopoietic progenitor cells;

b) genetically altering said cells by introducing into the genome of said cells at least one segment of DNA that encodes at least one protein capable of increasing intracellular production of glutathione in said cells; and

c) introducing said cells having increased intracellular production of glutathione into a patient for whom chemotherapy is prescribed, thereby enhancing the resistance of said cells and their progeny cells to dosages of said therapeutic agents that would be toxic to hematopoietic progenitor cells not genetically altered according to step (b) hereof.

2. A method as claimed in claim 1, wherein said at least one protein is selected from the group consisting of γ-glutamylcysteine synthetase, γ-glutamyltranspeptidase, glutathione synthetase, glutamine synthetase, γ-glutamylcyclotransferase, 5-oxyprolinase, glutathione peroxidase, glutathione reductase, glutathione transhydrogenase, or a combination thereof.

3. A method as claimed in claim 2, wherein said at least one protein comprises an enzymatically active form of γ-glutamylcysteine synthetase.

4. A method as claimed in claim 2, wherein said at least one protein comprises an enzymatically active form of γ-glutamyltranspeptidase.

5. A method as claimed in claim 2, wherein said at least one DNA segment comprises cDNA encoding said at least one protein.

6. A method as claimed in claim 2, wherein said at least one DNA segment comprises a gene encoding said at least one protein.

7. A method as claimed in claim 2, wherein said at least one DNA segment is introduced into said genome via retrovirus-mediated DNA transfer.

8. A method as claimed in claim 7, wherein said DNA transfer is accomplished by infection of said cells with an amphotrophic packaged replication-incompetent retroviral shuttle vector comprising said at least one DNA segment.

9. A method as claimed in claim 2, wherein said at least one DNA segment is introduced into said genome by a method selected from the group consisting of transfection via mammalian expression vector, via calcium phosphate precipitation, liposome-mediated transfer and electroporation-mediated transfer.

10. A method as claimed in claim 2, wherein expression of said at least one DNA segment is constitutive.

11. A method as claimed in claim 2, wherein expression of said at least one DNA segment is inducible.

12. A method as claimed in claim 2, wherein expression of said at least one DNA segment is under the control of a viral promotor.

13. A method as claimed in claim 2, wherein expression of said at least one DNA segment is under the control of a cellular promotor.

14. A method as claimed in claim 1, wherein said therapeutic agents comprise at least one alkylating chemotherapeutic agent.

15. A method as claimed in claim 14, wherein said at least one alkylating agent comprises a platinum-containing compound.

16. A method as claimed in claim 14, wherein said alkylating agent is selected from the group consisting of: cisplatin, carboplatin, tetraplatin, melphalan, cyclophosphamide and combinations or derivatives thereof.

17. A method as claimed in claim 1, wherein said therapeutic agents comprise anthrocyclines, epipodophilotoxins, vinblastine, radiation and combinations or derivatives thereof.

18. A method as claimed in claim 1, wherein said hematopoietic progenitor cells are provided by the steps of:

a) aspirating bone marrow from at least one histocompatible donor individual

b) optionally, further purifying from said aspirated bone marrow a cell population comprising said hematopoietic progenitor cells.

19. A method as claimed in claim 1, wherein said cells are introduced into said patient by infusion through said patient's bloodstream.

20. A method for increasing the resistance of hematopoietic progenitor cells to therapeutic agents used in treating a patient having a malignant tumor, said method comprising:

a) providing healthy hematopoietic progenitor cells;

b) genetically altering said cells by infection with a replication-incompetent retroviral shuttle vector comprising at least one cDNA encoding a biologically active protein capable of increasing intracellular glutathione production , such that said cDNA is stably incorporated into the genome of said infected cells, thereby imparting to said cells the capability of producing increased amounts of intracellular glutathione; and

c) introducing said genetically altered cells into said patient, so as to increase the resistance of said hematopoietic progenitor cells and their progeny to dosages of said therapeutic agents that would be toxic to hematopoietic progenitor cells not genetically altered according to step (b) hereof.

21. Recombinant hematopoietic progenitor cells for use in conjunction with therapeutic agents in treatment of patients having a malignant tumor,

said cells comprising a genome having at least one introduced segment of DNA that encodes at least one protein capable of increasing intracellular production of glutathione, resulting in said cells having enhanced resistance to concentrations of said therapeutic agents that would be toxic to non-recombinant hematopoietic progenitor cells.

22. Recombinant hematopoietic progenitor cells for use in conjunction with therapeutic agents in treatment of patients having a malignant tumor, said cells comprising a genome having at least one introduced segment of DNA that encodes an expressible, enzymatically active form of γ-glutamylcysteine synthetase capable of increasing intracellular production of glutathione, said cells expressing said γ-glutamylcysteine synthetase in an amount effective to substantially negate the inhibitory effect of a standard inhibitory dosage of buthionine sulfoximine, said cells having enhanced resistance to concentrations of said therapeutic agents that would be toxic to non-recombinant hematopoietic progenitor cells.

23. A method for treatment of a patient having a malignant tumor, comprising administering therapeutic agents, said method comprising the steps of:

a) infusing into said patient recombinant hematopoietic progenitor cells comprising a genome having at least one introduced segment of DNA that encodes an expressible, enzymatically active form of γ-glutamylcysteine synthetase capable of increasing intracellular production of glutathione, said cells expressing said γ-glutamylcysteine synthetase in an

amount effective to substantially negate the inhibitory effect of a standard inhibitory dosage of buthionine sulfoximine, said cells having enhanced resistance to concentrations of said therapeutic agents that would be toxic to non-recombinant hematopoietic progenitor cells; and

b) administering to said patient at least one of said therapeutic agents, at a dosage that would be toxic to said patient's non-recombinant hematopoietic progenitor cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02929

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00; C12N 5/00; A61K 49/00
US CL :435/172.3, 435/240.2, 424/93B

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 435/240.2, 424/93B;
935/11, 62, 111

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 4,497,796 (Salser et al.) 05 February 1985, see the entire document.	1-23
Y	The Journal of Biological Chemistry, Volume 265, No. 3, Issued 25 January 1990, Yan et al., "Amino acid sequence of rat kidney - glutamylcysteine synthetase", pages 1588-1593, see the entire document.	1-23
Y	NCI Monographs, Number 6, Issued 1988, Ozols et al., "Keynote address: mechanisms of cross-resistance between radiation and antineoplastic drugs", pages 159-165, see the entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

11 MAY 1993

Date of mailing of the international search report

23 JUN 1993

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Eur. J. Biochem., Volume 208, Issued 1992, Roemer et al., "Concepts and strategies for human gene therapy", pages 211-255, see the entire document.	1-23
Y	Nature, Volume 318, Issued 14 November 1985, Keller et al., "Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors", pages 149-154, see the entire document.	1-23
Y	Blood Cells, Volume 13, Issued 1987, Kohn et al., "Retroviral-mediated gene transfer into mammalian cells", pages 285-298, see the entire document.	1-23
Y	Proceedings of the National Academy of Sciences, USA, Volume 87, Issued May 1990, Bodine et al., "Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells", pages 3738-3742, see the entire document.	1-23
Y	Seminars in Oncology, Volume 18, No. 3, Issued June 1991, Ozols et al., "Chemotherapy of ovarian cancer", pages 222-232, see the entire document.	1-23

International application No.
PCT/US93/02929

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: DIALOG (Files 154, 55, 311, 312), USPTO Automated Patent System (File USPAT, 1975-1993)

Search terms: human, gene, therapy, transfer, review, resistance, tumor, cancer, GCS, synthetase, cloning, glutamylcysteine, ovarian, inventors' names